Week 13: 31. August 2015 – 4. September 2015

31. August 2015

1) Preculture for *in vitro* assay of MEDH2
   - Inoculate 5 ml LB+Kan [50 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.
   - Inoculate *E. coli* BL21 containing pET-28 selfligated or pET-28+*medh2*
2) Inoculate culture of *Methylococcus capsulatus*
   - Prepare a 30 ml liquid culture with NMS media containing 25 % (v/v) Methanol
   - Incubate at 42 °C shaking at 100 rpm
3) Digest synthesized *mmoG* with PstI and EcoRI
   - Pipetting scheme according to the protocol Restriction Digest
   - Incubate the Reaction at 37 °C for 1 hour
   - Verify 10 µl of the digestion on agarose gel

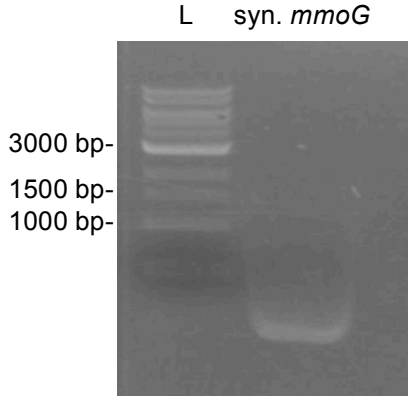

Figure 1: Digest of synthesized *mmoG* with PstI and EcoRI. 10 µl of digest were checked on 1% (w/v) agarose gel. Expected sizes: syn. *mmoG*- 1707 bp. As ladder (L) 1kB Ladder (NEB) was used.

1. September 2015

1) *In vitro* assay of NAD+ dependent methanol dehydrogenase
   - Use as negative control: *E. coli* strain containing the selfligated pET-28 vector
   - Inoculate a mainculture (50 ml) and induce protein expression by adding IPTG to a final concentration of 1 mM at an OD_{600} of 0.4- 1.0

Table 1: OD_{600} at time point of induction

Sample	**OD_{600}**
Negative control	0,576
MEDH2 Exp Culture	0,789

- Incubate at 37 °C for 3 hours
- Harvest the culture by centrifugation at 8000 x g for 5 minutes
- Resuspend the cell pellet in 1 ml 50 mM K_2HPO_4 buffer and add 10 µl of Halt™ Protease Inhibitor Single-Use Cocktail (100X) (ThermoScientifc)
- Add glass beads (0.1 mm diameter) and disrupt the cells by shaking for 12 minutes
- Centrifugation at 16000 x g for 5 minutes
- Keep the soluble fraction for the assay
- Perform the enzyme assay in a total volume of 1 ml:
    - Preheated 50 mM K_2HPO_4 + 5 mM $MgSO_4$ Buffer
    - Use 50 µl soluble fraction
    - Add methanol to a final concentration of 1 M (Stock 10M)
    - Detect the absorption at 340 nm over 1 hour
    - Measurements are performed in triplicates
- Blank against enzyme assay reaction mix containing 50 µl of soluble fraction of the negative control

	Absorption at 340 nm	
Time in min	Control without addition of Methanol	Addition of Methanol
0	0,0515	0,701
1	0,0638	0,977
2	0,0746	1,469
3	0,0835	2,435
4	0,0927	3,148
5	0,0999	3,833
6	0,1064	4,409
7	0,1119	4,93
8	0,1161	5,442
9	0,1195	5,763
10	0,1223	6,151
11	0,1251	6,396
12	0,1271	6,655
13	0,1293	6,846
14	0,1315	7,08
15	0,1334	7,255
16	0,1357	7,397
17	0,1378	7,527
18	0,1401	7,634
19	0,1422	7,744
20	0,1443	7,804

21	0,1464	7,869
22	0,1488	7,927

- Preculture for *in vivo* assay of MEDH2
- Inoculate 5 ml LB+Kan [50 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.
- Inoculate *E. coli* BL21 containing pET-28 selfligated or pET-28+*medh2*
- PCR to amplify synthesized *mmoG* for BioBrick System
- Pipetting scheme and PCR program according to PCR with Phusion-HF DNA Polymerase protocol
- Primer: mmoGB-BioBrick-FWD/Rev
- Template: synthesized *mmoG* (26. June 2015)
- Analyse 10 µl of PCR reaction on 1 % agarose gel
- Failed

2. September 2015

***In vivo* assay of NAD+ dependent methanol dehydrogenase**

- Use as negative control: *E. coli* strain containing the selfligated pET-28 vector
- Inoculate a mainculture (50 ml), containing Kanamycin [50µg/ml] and add 0.1 mM IPTG, measure OD_{600}

Table 2: OD600 of main culture after inoculation. Numbers 1-3 determine three different expression cultures of MEDH2 all derived from the same clone.

Sample	**OD_{600}**
Negative Control	0.362
Expression culture 1	0.481
Expression culture 2	0.301
Expression culture 3	0.383

- Grow the culture until the stationary phase is reached.
- Measure OD600 of the culture and harvest a cell pellet of an OD600 of 1 in a volumne of 50 ml by centrifugation at 8000 x g for 10 minutes at roomtemperature
- Resuspend the cell pellet in 50 ml M9 minimal media and measure the actual OD_{600}

Table 3: Actual OD600 before starting i*n vivo* assay

Sample	**OD_{600}**
Negative Control	0.822
Expression culture 1	0.925
Expression culture 2	0.936
Expression culture 3	0.933

- Take a 500 µl sample of each culture which will serve as a blank
- Start the experiment by adding 2 ml of 10 % (v/v) methanol
- Directly after adding the methanol take a 500 µl sample
- Take a 500 µl sample every 5 minutes over a 1 hour.
- Pellet the cells by centrifugation at 11000 x g for 5 minutes at 4 °C
- Use 500 µl of the supernatant with 500 µl of Nash reagent and measure absorption at 412 nm and determine the concentration of formaldehyde

	Absorption at 412 nm			
Time in min	**NC**	**1**	**2**	**3**
0	0,0091	0,0101	0,0831	0,0069
5	0,0129	0,001	-0,0269	0,0117
10	0,0697	0,206	0,0074	0,0013
15	0,0095	0,1422	0,0183	0,1185
20	-0,017	-0,0104	-0,0022	-0,083
25	-0,0277	0,0132	-0,0266	-0,0007
30	0,0026	-0,0127	-0,0201	-0,0116
35	-0,0167	0,0057	-0,0029	0,0054
40	0,3419	0,4082	0,3865	0,3849
45	-0,0168	-0,4034	-0,0116	-0,0079